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PII: S0306-4530(15)00184-5
DOI: http://dx.doi.org/doi:10.1016/j.psyneuen.2015.05.004
Reference: PNEC 2993

To appear in:

Received date: 1-2-2015
Revised date: 16-4-2015
Accepted date: 8-5-2015

Please cite this article as: Mina, T.H., Räikkönen, K., Riley, S.C., Norman, J.E., Reynolds, R.M.,Maternal Distress Associates with Placental Genes Regulating Fetal Glucocorticoid Exposure and IGF2: Role of Obesity and Sex, *Psychoneuroendocrinology* (2015), http://dx.doi.org/10.1016/j.psyneuen.2015.05.004

Highlights

- Elevated maternal emotional distress symptoms during pregnancy are associated with changes in placental genes regulating fetal glucocorticoid exposure and placental growth
- Although severely obese women have increased emotional distress symptoms compared to normal-weight our findings suggest a protective adaptive responses from excess glucocorticoid exposure in the placentas of severely obese pregnancy
- The observed changes in placental gene expression suggest female fetuses are more vulnerable to maternal distress than males with potentially greater fetal glucocorticoid exposure and excess IGF2
- Further studies are needed to understand the placental adaptive changes to maternal distress in obese pregnancy and whether thee findings translate to potentially greater negative outcomes of maternal distress in female offspring in early childhood.

Maternal Distress Associates with Placental Genes Regulating Fetal Glucocorticoid Exposure and IGF2: Role of Obesity and Sex

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Running title:

Maternal distress and placental gene expression

Abstract

Maternal emotional distress symptoms, including life satisfaction, anxiety and depressed mood, are worse in Severely Obese (SO) than lean pregnancy and may alter placental genes regulating fetal glucocorticoid exposure and placental growth. We hypothesised that the associations between increased maternal distress symptoms and changes in placental gene expression including IGF2 and genes regulating fetal glucocorticoid exposure are more pronounced in SO pregnancy. We also considered whether there were sex-specific effects. Placental mRNA levels of 11β-HSDs, NR3C1-α, NR3C2, ABC transporters, mTOR and the IGF2 family were measured in term placental samples from 43 lean (BMI ≤25 kg/m^2) and 50 SO (BMI ≥40 kg/m^2) women, in whom distress symptoms were prospectively evaluated during pregnancy. The mRNA levels of genes with a similar role in regulating fetal glucocorticoid exposure were strongly inter-correlated. Increased maternal distress symptoms associated with increased NR3C2 and IGF2 isoform 1(IGF2-1) in both lean and SO group ($p \leq 0.05$). Increased distress was associated with higher ABCB1 and ABCG2 mRNA levels in SO but lower ABCB1 and higher 11β-HSD1 mRNA levels in lean ($p \leq 0.05$) suggesting a protective adaptive response in SO placentas. Increased maternal distress associated with reduced mRNA levels of ABCB1, ABCG2, 11β-HSD2, NR3C1-α and IGF2-1 in placentas of female but not male offspring. The observed sex differences in placental responses suggest greater vulnerability of female fetuses to maternal distress with potentially greater fetal glucocorticoid exposure and excess IGF2. Further studies are needed to replicate these findings and to test whether this translates to potentially greater negative outcomes of maternal distress in female offspring in early childhood.

Key Words

obesity, pregnancy, anxiety, depression, life satisfaction, placenta, glucocorticoids, ABC transporters, 11b-dehydrogenase, IGF2

1. Introduction

Obesity and emotional distress symptoms such as anxiety and depression (A&D) are prevalent during pregnancy (Gavin et al., 2005, Heslehurst et al., 2010). Both independently associate with increased obstetric complications, adverse birth outcomes (Alder et al., 2007) postpartum depression (Molyneaux et al., 2014), and also poor infant health (Lawlor et al., 2012), cognitive and behavioural development (Van Lieshout et al., 2011).

One of the key pathways linking maternal obesity and A&D with adverse pregnancy outcomes is altered maternal hypothalamic-pituitary-adrenal (HPA) axis activity (Lopresti & Drummond, 2013). Increased glucocorticoid exposure during pregnancy in association with maternal A&D has been associated with cognitive and behavioural problems (Mina & Reynolds, 2014). It is also strongly linked to low birth weight, leading to increased metabolic and cardiovascular risk in later life (Reynolds, 2013). We previously demonstrated that maternal very severe obesity (SO, WHO obese class III, BMI $\geq$ 40 kg/m^2) is independently associated with antenatal A&D symptoms (Mina et al., 2015), emphasising the importance of co-investigating both conditions to further understand their adverse impact on fetal outcome and future infant health.

Changes in the maternal HPA axis during gestation promote increased glucocorticoids (Mastorakos and Ilias, 2003), which are important for fetal lung maturation. The placenta modulates fetal glucocorticoid exposure, but is also developmentally plastic and is therefore responsive to insults. The fetus is protected from excess maternal cortisol by placental 11 beta-hydroxysteroid dehydrogenase (11β-HSD) 2 (Chapman et al., 2013), and down regulation of 11β-HSD2 has been associated with increased maternal anxiety (O'Donnell et al., 2012) but not depression (Ponder et al., 2011, O'Donnell et al., 2012, Reynolds et al., 2015). No studies have linked maternal A&D to placental 11β-HSD1 (Reynolds et al., 2015),

which catalyses the regeneration of active glucocorticoids. We recently showed that maternal depressive symptoms associate with increased mRNA levels of placental glucocorticoid receptor (NR3C1) and mineralocorticoid receptor (NR3C2) (Reynolds et al., 2015). This, and the observation of links between maternal A&D and altered methylation of placental NR3C1 (Conradt et al., 2013), implies altered placental sensitivity to glucocorticoids in association with maternal A&D. A further level of control of fetal glucocorticoid exposure is through retrograde transfer of glucocorticoids from placenta to mother via the placental ATP- Binding Cassette (ABC) transporter family (apical ABCB1 and ABCG2, basal ABCC1). Whether mRNA levels of these transporters are associated with maternal A&D has not been investigated.

In animal models maternal prenatal stress also increases expression of placental Insulin- like Growth Factor (IGF) 2 (Pankevich et al., 2009), a key factor regulating placental and fetal growth and development (Burton and Fowden, 2012). Placental IGF2 expression is also vulnerable to maternal obesity, though varies with gestation. For example, increased mRNA levels of *igf2* at day 16 of pregnancy were observed in placentas from the fetuses of dams fed with high-fat diet during pregnancy as compared to dams with standard diet, although this effect was no longer observed at day 19 of pregnancy (Sferuzzi-ferri et al., 2013). Preliminary data suggests that *igf2* mRNA levels are modulated by prevailing glucocorticoid levels (Vaughan et al., 2012) and may be sex-specific with up-regulation of placental *igf2* transcription by glucocorticoids in placentas of male, but not in female mice offspring (Cuffe et al., 2012). Mammalian Target of Rapamycin (mTOR), an intracellular nutrient stress sensor in the placenta (Roos et al., 2009) has been linked to the downstream signalling of both glucocorticoids (Jellyman et al., 2012) and IGF2 (Dai et al., 2011). A small study reporting a lower placental DEP domain-containing mTOR-interacting protein (DEPTOR) in mothers with higher perceived life stress and reduced DEPTOR following

increased cortisol *in vitro* (Mparmpakas et al., 2012) suggests further investigation of these genes is warranted.

In this study, we aimed to evaluate the effects of both maternal obesity and A&D symptoms on placental mRNA levels of genes regulating fetal glucocorticoid exposure and the IGF2 family. We hypothesised that increased maternal emotional distress symptoms would be associated with changes in placental gene expression leading to a reduced placental barrier to excess maternal glucocorticoid levels and increased IGF2 mRNA levels. We speculated that this would be more pronounced in maternal obesity due to the greater levels of distress symptoms compared to lean (Mina et al., 2015). We considered whether there were sex- specific changes in placental gene expression in association with maternal distress. To test this hypothesis we used placental samples collected at term from SO women and lean controls participating in a longitudinal case-control study of obesity in pregnancy and in whom maternal A&D symptoms and serum cortisol levels were assessed prospectively during pregnancy (Mina et al., 2015).

2. Materials and Methods

2.1. Participants

This study included women with singleton pregnancies participating in a prospective case-control study comparing lean (BMI ≤25 kg/m^2) and SO (BMI ≥40 kg/m^2) women in Edinburgh, Scotland, UK. As previously described, SO women were recruited from a specialist antenatal metabolic clinic for women with BMI>40 kg/m^2) whilst lean women were recruited from community antenatal clinics (Mina et al., 2015). Maternal body composition, maternal and pregnancy factors were characterised (Mina et al., 2015). All samples and data were collected with ethical approval (references 08/S1101/39 and 13/ES/0126), and with fully informed and written consent from all women.

2.2. Assessments of emotional distress and measurement of serum cortisol

Maternal distress was assessed at week 17 and at week 28 of gestation (Mina et al., 2015). Briefly, the assessment comprised 5 previously validated self-rating items in printed questionnaires: 1) psychosocial risk factor assessment (Rosengren et al., 2004); 2) Satisfaction with Life Scale (SWLS, Diener et al., 1985); 3) General Health Questionnaire (GHQ, Goldberg 1972); 4) Hospital Anxiety and Depression Scale (HADS, Zigmond & Snaith 1983) and 5) State-Trait Anxiety Index (STAI, Spielberger 1927). A single question "*Have you consulted your General Practitioner (GP) about mental health issues in the last 2 years?*" was included.

Serum cortisol levels were measured in maternal samples collected at 9am in the morning following an overnight fast during the first clinic visit (week 17), week 28 and 36 of pregnancy, as previously described (Stirrat et al., 2014, Mina et al., 2015).

2.3. Placental tissue collection, RNA extraction and quality assessment

Placental tissues were taken as single biopsies from the maternal side of the placenta and were stored in RNAlater solution (Qiagen, Manchester, UK) at 4˚C for 24 hours and subsequently frozen at -80˚C. A set of 93 placental samples (43 lean and 50 SO) were randomly selected after excluding samples from participants with gestational diabetes mellitus, treated with antenatal steroids during pregnancy and preterm births. For RNA extraction, approximately 30 mg of frozen placental tissues was excised and snap-frozen into Magnalyser green beads (Roche, Burgess Hill, UK). Each sample was subsequently submerged in 500 µl buffer RLT (part of Qiagen RNeasy Fibrous Tissue Mini kit) with additional β-mercaptoethanol (10:1), then homogenised with Qiagen Tissue Lyser for 50 seconds x 25 Hz for 4-5 times with brief ice interruption to keep the Magnalyser tubes cool. Following tissue lysis, lysates were processed as per the Qiagen RNeasy Fibrous Tissue Mini kit protocol. RNA concentration was quantified by Nanodrop (Thermo-Scientific, Loughborough, UK). RNA integrity was evaluated by Agilent 6000 Nano eukaryotic total RNA assay (Agilent, Berkshire, UK).

2.4. Primer design and validation, cDNA synthesis and Quantitative Real Time PCR (QRTPCR)

Placental cDNA was synthesised with ABI High Capacity Reverse Transcriptase kit (Life Technologies, Paisley, UK) as per the manufacturer's instruction into 500 ng/µl end-product.

Exon-exon junction spanning- primers and probe pairs for FAM hydrolysis were designed using ProbeFinder v2.50 for ABCB1, ABCC1, ABCG2, 11β-HSD1, 11β-HSD2, NR3C1-α, NR3C2, IGF2 isoform 1(IGF2-1), IGF2 isoform 2 (IGF2-2), IGF2 receptor (IGF2R), and mTOR (Roche, **A Table 1**). Primers were validated *in silico* with PrimerBLAST (NCBI) and *in vivo* with conventional PCR using reagents and protocol from GoTaq®2 DNA polymerase mixture (Promega, Southampton, UK). The conventional PCR product was run in 2% agarose gel with 1X gel-red (Biotium, Cambridge, UK) along with 100-bp DNA ladder (Trackit^{TM}, Invitrogen, Life Technologies) in 0.5x TBE buffer to assess probe specificity.

For absolute QRTPCR, all samples were assayed in triplicates using FAM hydrolysis thermal cycling set-up in LightCycler 480 QRTPCR system (Roche), and housekeeping genes assay was performed for each batch of cDNA synthesis. A standard curve was prepared by pooling 1µl of each cDNA sample, which was diluted 1:4 with RNase-free water, and then serially diluted 1:2 to produce 8 standards (1:4- 1:512). The QRTPCR reaction mixture was prepared following PerfeCTa® QRTPCR Fastmix® II (Quanta Biosciences, VWR, Leicestershire, UK) protocol, with final primer concentration = 250 nM, probe = 100 nM and cDNA per reaction = 25 ng. The datasets were analysed with LightCycler 480 QRTPCR software using E-Methods, and only those with standard error ≤0.05 and efficiency ≥1.7 were considered for further analysis (**A Table 2)**. Two housekeeping genes suitable for placental tissue (YWHAZ and TBP, Meller et al., 2005) were utilised.

2.5. Covariates, Confounders and Moderators

Age, parity, deprivation category, alcohol and cigarette consumption before pregnancy, use of anti-depressants, history of polycystic ovarian syndrome (PCOS), inflammatory illnesses, and minor obstetric complications were assessed by questionnaire and verified through search

of hospital records. Delivery outcomes such as birth weight, mode of delivery, fetal sex, and gestational age were extracted from hospital records.

2.6. Statistical Analyses

Statistical analyses were performed using SPSS 19 (IBM, New York, USA). $P \leq 0.05$ was used as a cut-off of statistical significance. Prior to any analyses, data distribution was determined by Q-Q Plot and by histogram visualisation. The gene expression data were first normalised using an average of YWHAZ and TBP housekeeping genes and then log-transformed. For the distress questionnaires there were missing data due to either improper completion of the questionnaire or failure to complete an entire scale. Overall there were 2.3 % missing distress assessment data in lean and 12% in the SO group. Missing data imputation was performed on these data using Markov Chain Monte Carlo (MCMC) algorithm.

Pearson's correlations were used to test intra- and inter-correlation among the genes. Any non-linear correlations were detected using curve-fitting method. To avoid multiple statistical testing when interpreting the A&D questionnaire data, we restricted our analysis to include 'state anxiety' (as this differed between SO and lean) and used the averaged z-scores of either "anxiety symptoms" (Hospital Anxiety (HA) from HADS, State and Trait Anxiety) or "depression symptoms" (Hospital Depression (HD) from HADS and GHQ) (as in Mina et al., 2015). Serum cortisol level at week 36 pregnancy was selected as it was the nearest to the delivery.

Due to the large numbers of variables examined, a set of covariates and confounders was constructed for each of the lean, SO, male and female placental pool by testing the correlations of the genes with variables which either differed between SO and lean groups (Mina et al., 2015), or have been shown to greatly influence placental gene expression (Burton and Fowden 2012, Burton et al., 2014, Räikkönen et al 2014, Reynolds et al., 2015)

(**A Table 3**). We also tested whether fetal sex was a potential confounder for lean and SO pool, and vice versa (**A Table 3**). Multiple linear regressions with step-down methods were subsequently applied to assess whether maternal emotional distress symptoms were associated with gene mRNA levels in the SO group and separately in the lean group. We then repeated the analyses in the male and female offspring, respectively.

Power calculation was performed using GPower 3.1 with command [F-Test, Linear Multiple Regression: Fixed model, R^2 increase, *a priori* mode]. We used 70% and 90% as minimum and maximum power, respectively, with $\alpha = 0.05$. Mood symptoms at week 17 were taken as reference. Random integer generator (http://www.random.org/integers/) was used to determine 2 representative genes, and where applicable comprised both significant and not significant associations. Analyses were performed in each BMI group and fetal sex group. The power for all maternal SO and fetal sex analyses was >90%, except for the associations involving anxiety symptoms in male group (<70%).

3. Results

3.1. The demographics of participants

Table 1 presents the demographics of participants (lean n= 43, SO n= 50) and the details of delivery mode and birth outcomes. SO group had higher maternal emotional distress symptoms and lower serum cortisol levels as compared to the lean women, consistent with the cohort's findings (Mina et al., 2015). There were significantly higher numbers of caesarean deliveries in SO group as compared to lean. In this randomly selected sample there was a significant difference in the sex composition with more males from SO women.

3.2. Correlations among glucocorticoid-linked genes and IGF2 family

Table 2 presents the inter-correlation of mRNA levels among the placental genes. The genes which either facilitate (ABCC1, 11βHSD1) or prevent (ABCB1, ABCG2, 11βHSD2) fetal glucocorticoid exposure were positively correlated. The mRNA levels of the glucocorticoid receptor NRC31-α were positively correlated with genes facilitating fetal glucocorticoid exposure, but negatively correlated with genes preventing fetal glucocorticoid exposure.

IGF2 isoforms, IGFR and mTOR were positively correlated. IGF2-2 isoform was not transcribed as much as IGF2-1 isoform (**A Fig 1**), consistent with previous findings (De Ceuninck et al., 1995).The mRNA levels of the IGF2 family and mTOR were also positively correlated with genes facilitating fetal glucocorticoid exposure (NR3C1-α, ABCG2), negatively correlated with ABCB1, and not with 11β-HSD2. The mRNA levels of NR3C2 did not correlate with any of the studied genes (**Table 2**).

In unadjusted analyses there were no significant linear associations between cortisol levels and mRNA levels of the tested genes. However, after adjustment for confounders including maternal age, antidepressant use during pregnancy, fetal sex and deprivation category, lower serum cortisol level at week 36 pregnancy was associated with increased NR3C2 mRNA levels in the SO group (β=-0.42, p= 0.03), while increased serum cortisol level was associated with increased ABCC1 mRNA levels in the lean group (β=0.37, p=0.017). There was an inverted U-shaped correlation between 11β-HSD2 mRNA levels and serum cortisol levels in total sample pool such that 11β-HSD2 levels were lowest at both low and high cortisol levels (R^2= 0.11, p = 0.03, **A Fig 2**). Although there were no differences in serum cortisol levels according to fetal sex, some sex-specific associations between serum cortisol and placental mRNA levels were observed. In placentas with female fetuses, increased serum cortisol level was associated with higher ABCC1 (β= 0.52, p≤ 0.05), 11β-HSD1 ((β= 0.46, p≤ 0.05) and IGF2-1 (β=0.35, p≤ 0.05) mRNA levels. In placentas with male fetuses, increased serum cortisol was associated with lower 11β-HSD1 (β=-0.48, p≤ 0.05) and NR3C1-α (β= -0.32, p≤ 0.05) mRNA levels.

3.3. Placental glucocorticoid-linked and IGF2 family mRNA levels associate with maternal emotional distress symptoms in both obese and lean

In the total sample pool regardless of maternal obesity status, increased maternal distress at week 28 pregnancy was correlated increased mRNA levels of IGF2-1 (r=0.28, p≤ 0.01 with reduced SWLS) and reduced IGF2-2 (r=–0.26 with anxiety symptoms and r= –0.21 with depression symptoms, all p≤ 0.05). There were no differences in the mRNA levels of any of

the genes tested according to maternal obesity status except for lower ABCB1 mRNA levels in SO compared to lean in adjusted analyses (β= –0.361, p= 0.019).

In the SO group, lower perceived life satisfaction (higher emotional distress) at week 17 of pregnancy was associated with higher ABCG2 (β= -0.46, p≤ 0.05) and with higher NR3C2 (**Fig 1A**) mRNA levels. Lower life satisfaction at week 28 pregnancy in SO group was also associated with higher IGF2-1 gene expression (**Fig 1B**). A similar pattern was observed in the lean group where increased state anxiety at week 17 pregnancy (higher emotional distress) was associated with higher mRNA levels of NR3C2 (**Fig 1A**) and IGF2-1 (**Fig 1B**).

In the SO group, lower perceived life satisfaction at week 17 pregnancy was associated with higher ABCB1 (**Fig 1C).** In contrast in lean, lower life satisfaction in early (week 17) and late (week 28) pregnancy was associated with decreased ABCB1 mRNA levels (**Fig 1C** and β= 0.34, p≤ 0.05, respectively). Furthermore, increased anxiety symptoms at week 17 pregnancy were associated with increased 11β-HSD1 mRNA levels (β= 0.35, p≤ 0.05) in lean, but not in the SO group.

There were no associations of maternal A&D symptoms or satisfaction with life with the mRNA levels of 11β-HSD2, NR3C1-α, IGF2-2, IGF2R and mTOR in either group.

3.4. The changes of glucocorticoid-linked and IGF2 family placental genes with increased maternal emotional distress symptoms are sex-specific

Regardless of maternal SO status, the mRNA levels of some placental genes differed significantly according to fetal sex (NR3C1 [p<0.001], NR3C2 [p= 0.05], IGF2-2 [p= 0.05], IGF2-R [p= 0.02] were all higher in females). Fetal sex also emerged as an important

confounder for some genes during the obesity-based analyses. Analysing the data separately for males and females revealed sex differences in the associations between placental gene expression and maternal emotional distress symptoms (**Table 3**). In pregnancies with female fetuses, mRNA levels of genes which prevent glucocorticoid transfer to the fetuses were lower (ABCB1, ABCG2, 11βHSD2, **Table 3**) in women with increased emotional distress symptoms (increased A&D symptoms or decreased SWLS). In female placentas the mRNA levels of NR3C1-α were also higher in association with lower perceived life satisfaction (higher emotional distress) (**Table 3**). In contrast, in male placentas, lower perceived life satisfaction was associated with increased mRNA levels of genes which prevent glucocorticoid transfer to the fetuses (ABCG2, 11βHSD2, **Table 3**), suggesting a protective effect. After additional adjustment for serum cortisol level at week 36 pregnancy, reduced life satisfaction was also associated with increased 11βHSD1 in males (β=-0.39, $p \leq 0.05$).

Table 3 also demonstrates that increased maternal emotional distress symptoms were associated with increased IGF2-1 and with trends for increased IGF2R mRNA levels in female, but not in male placentas. In male placentas, increased state anxiety in week 17 was associated with reduced IGF2-2 mRNA level (β= -0.45, $p \leq 0.05$). The associations involving IGF2-1 in female placentas were no longer significant when adjusted for serum cortisol level at week 36 pregnancy. No significant associations were found in ABCC1, NR3C2, mTOR mRNA levels with maternal A&D symptoms in either sex. Overall, **Table 3** suggests that female placentas were more responsive to increased maternal emotional distress symptoms.

4. Discussion

Our findings demonstrate that maternal emotional distress associates with changes in mRNA levels of several placental genes involved in the regulation of fetal glucocorticoid exposure and placental growth. Contrary to our hypothesis we found that the pattern of changes in mRNA levels in placentas from SO women (who had greater A&D symptoms than lean), were largely similar to those in placentas from lean women. However, in exploratory analyses we observed sex-specific responses, with placentas with female fetuses appearing more vulnerable to maternal A&D symptoms than placentas with males.

To our knowledge, no other studies using human placentas have investigated whether mRNA levels of glucocorticoid-linked or the IGF2 family genes vary according to maternal obesity. Despite marked differences in obesity levels between our study groups, only the mRNA levels of ABCB1 significantly differed, with lower levels in placentas from SO than lean women. The physiological relevance of this observation is not known as placental ABCB1 levels are lowest at late stages of pregnancy (Sun et al 2006), but as ABCB1 actively pumps various xenobiotics and glucocorticoids from the fetal to the maternal circulation (Kalabis et al., 2005), the lower ABCB1 levels in SO may partly explain the lower maternal circulating cortisol levels. Although there are no studies in placenta, global deficiency of ABCB1 appears to be associated with susceptibility to developing obesity in rodent models, since feeding Pgp (*abcb1*) knock-out mice with a high-fat diet induced obesity (Foucaud-Vignault et al., 2011). Likewise, in humans, a polymorphism of ABCB1 influencing transcription and function is associated with obesity (Ichihara et al., 2008). Whether there is a genetic susceptibility to decreased ABCB1 activity in this cohort of SO women is unknown. Notably, the observations of increased placental *igf2*, *nr3c1* and *igf2r* in rodent models receiving either an obesogenic diet during pregnancy (Sferuzzi-ferri et al., 2013) or a chronic

obesogenic diet (King et al 2013) were restricted to mid-gestation with no differences at term. If there are similar transient changes in mRNA levels in mid gestation in human placenta, this may explain the lack of differences between lean and SO in our study using samples collected at term.

The pattern of changes in placental mRNA levels, other than ABCB1, with maternal distress was similar in lean and SO. For example, increased maternal emotional distress, as reported by increased state anxiety scores or reduced scores on the SWLS, was associated with increased mRNA levels of NR3C2 and IGF2-1 in both groups. This is similar to the increased placental mRNA levels of NR3C2 in association with maternal depressive symptoms in another cohort of pregnant women (Reynolds et al., 2015), a finding consistent with increased placental sensitivity to glucocorticoids in situations of maternal distress. The IGF2-1 findings are novel but differ from a study reporting no significant associations between IGF2 DNA methylation and diagnosis of maternal depression in pregnancy (Liu et al., 2012). This discrepancy may be due to our focus on a spectrum of maternal emotional distress symptoms rather than a clinical diagnosis of a psychiatric disorder. We also observed increased 11β−HSD1 mRNA level following increased state anxiety in lean, a similar finding to that observed in mothers with lower socio-economic status (Raikkonen et al., 2014), another strong indicator of maternal wellbeing (Heslehurst et al., 2010). This is in accord with literature supporting increased fetal glucocorticoid exposure with increased maternal distress during pregnancy (Duthie and Reynolds, 2013). Indeed, the placental glucocorticoid barrier in lean seems more sensitive to maternal distress as lower maternal distress in lean placentas was also associated with lower ABCB1 mRNA levels, implying more retrograde transfer of glucocorticoids back to the maternal circulation. Furthermore, contrary to our hypothesis, our data also suggest a paradoxically improved placental glucocorticoid barrier following increased maternal distress in SO placentas since first, the mRNA levels of 11β-

HSD1 in SO group remained unaltered unlike in lean, and second the ABCB1 and ABCG2 mRNA levels, both of which transport glucocorticoids from fetus to mother, were higher following reduced life satisfaction.

The marked differences in gene expression according to fetal sex suggest that sex dimorphism modulates placental responses to maternal A&D symptoms at the transcriptional level. We acknowledge that these findings need to be interpreted with caution, particularly as there was a difference in sex composition of the placental samples obtained from SO and lean women, but the observed changes in gene expression according to fetal sex remained significant after adjustment for maternal obesity. Human studies investigating the impact of maternal A&D on gene expression and/or epigenetic changes of glucocorticoid-linked genes in placenta and cord blood have either adjusted for sex or have not reported any sex difference (Oberlander et al., 2008, Ponder et al., 2011, O'Donnell et al., 2012, Raikkonen et al., 2014; Reynolds et al., 2015). Only one study highlighted the lower methylation of IGF2 in low birth weight female infants from mothers with depression (Liu et al., 2012), and accordingly we observed higher mRNA levels of IGF2-1 in placentas from females in association with increased maternal A&D symptoms. Further studies are needed to replicate these findings and follow-up of this cohort is needed to test whether these findings will translate into sexual dimorphism of stress reactivity (Davis & Emory, 1995) or behaviours (Connellan et al., 2000) in infant and childhood.

Given the pivotal role of placental 11β-HSD2 in regulating fetal glucocorticoid exposure, we were surprised that it did not associate with maternal obesity, A&D symptoms or serum cortisol level. A previous study which showed associations between maternal anxiety and down-regulation of 11β-HSD2 (O'Donnell et al 2012), included assessment of maternal mood on the day before caesarean delivery, which may have influenced the state

anxiety responses to the questionnaire, whereas we evaluated mood symptoms prospectively. We previously reported non-linear correlations between maternal anxiety and serum cortisol level across pregnancy (Mina et al., 2015) and the non-linear correlation of serum cortisol with 11β-HSD2 observed here may have masked any findings. In addition, the existing literature in this field is conflicting since synthetic glucocorticoids have been reported to increase 11β-HSD2 abundance (van Beek et al., 2004) whilst increasing endogenous cortisol at time of labour associated with decreased 11β-HSD2 abundance (Murphy and Clifton, 2003).

Importantly our study demonstrated that the prevailing level of circulating cortisol in late pregnancy may influence placental mRNA levels. For example, the associations of cortisol levels with the mRNA levels of 11β-HSD1 in male placentas and with ABCC1 and 11β-HSD1 in female, are in line with increased glucocorticoid transport to the fetus following increased maternal distress. The changes in the significance of the associations between IGF2-1 mRNA level and maternal distress in female pool after adjusting for serum cortisol level also suggest that glucocorticoids are one of the factors mediating the impact of maternal distress on placental gene expression. Further studies are needed to explore this further, to examine cortisol measurements nearer the time of delivery and/or measurements of cortisol in cord blood, and to examine the role of other potential biological agents, such as inflammatory cytokines.

The main strength of our study is the prospective study design and the well-defined case-control cohort with detailed characterisation in pregnancy, enabling us to adjust for an array of confounders. The subset of random samples that were selected for analysis were representative of the larger cohort (Mina et al., 2015) but were not complicated by cases of pre-term birth, antenatal steroids and gestational diabetes. We comprehensively assessed

placental genes regulating fetal glucocorticoid exposure including not only genes regulating glucocorticoid clearance and activation, but also genes involved in glucocorticoid sensitivity and transport across the placenta. Notably we found correlations among glucocorticoid-linked and IGF2 gene family according to whether they facilitate or prevent fetal glucocorticoid exposure, suggesting synergistic regulation of the placental glucocorticoid barrier. We found no changes in the transcriptional level of mTOR by maternal obesity, A&D symptoms or fetal sex. Increased activation of mTOR complex has been correlated with cases of macrosomia in obese pregnancy (Jannsson et al., 2013) and further work should consider the mTOR signalling pathway

A limitation of our study is the lack of protein and/or enzyme activity data, and that our work in term placenta could inevitably only provide a snapshot of events at the end of pregnancy at the only time the tissue can be collected. We also could not adjust for fetal cortisol levels in our analyses as cord blood and/or amniotic fluid samples were unavailable. However, we included adjustment for maternal serum cortisol level at week 36 pregnancy as it was the sample collected nearest to the time of delivery, and since maternal plasma cortisol levels are significantly correlated with cord plasma cortisol levels (Sybulski et al., 1975). The lower cortisol levels in our SO women were consistent with findings in overweight women (Wright et al., 2013) but further studies with more detailed assessment of cortisol during pregnancy and peripartum are needed. Of note, the inclusion of adjustment for maternal cortisol did impact on some of the findings. This is in accord with the changes in the mRNA levels of ABCB1, 11β-HSD2 and NR3C1-α following the modulation of serum glucocorticoids in healthy rat (Mark et al., 2009), mice (Cuffe et al., 2012) and in human placentas taken from women receiving antenatal steroids with small-for-gestational-age infants (Hodyl et al., 2013). Finally we acknowledge that the sex composition of the placentas in the SO group is significantly different from the controls and while we adjusted

for maternal obesity in the statistical analysis, the sex differences we observed require replication.

In conclusion, we observed changes in placental genes regulating fetal glucocorticoid exposure and IGF2 in association with maternal emotional distress symptoms during pregnancy. The pattern of changes suggest placentas from lean are sensitive to maternal distress while there is a protective adaptive response in the placentas from SO. In addition, the observed sex differences in responses are consistent with potentially greater fetal glucocorticoid exposure and excess IGF2 in female fetuses following increased maternal A&D symptoms, suggesting greater vulnerability of females to maternal mood. Further studies are needed to replicate these findings and to test whether this translates to potentially greater negative outcomes of maternal A&D symptoms in females in early childhood.

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Figure and Table Captions

Figure 1

Messenger RNA levels of glucocorticoid-related and IGF2-2 genes in the placenta according to maternal obesity. SO= Severely Obese. Life satisfaction refers to the SWLS scores (Satisfaction with Life Scale), in which the higher the score the better the wellbeing. For illustration purpose, both the X-axis of life satisfaction and the associated β values (β^R) were reversed. Y- Axis = log of normalised gene expressions against housekeeping genes. β= adjusted for the list of confounders for lean and SO group respectively, as defined **in S Table 3**. The mRNA levels of NR3C2 in SO group is additionally adjusted for serum cortisol level at week 36 of pregnancy. ……95% confidence interval.

Table 1

The demographics of participants, SO= Severely Obese. Bold italic: $p \leq 0.05$, underlined $p \leq 0.1$. [a] student's t-test, [b] Fisher's exact test, [c] Chi-square test. PCOS= Polycystic Ovarian Syndrome, HA= Hospital Anxiety, HD= Hospital Depression, GHQ= General Health Questionnaire. Averaged z-scores are derived from the z-scores of HA, State and Trait for anxiety symptoms and HD and GHQ only for depression symptoms. [1] Deprivation category was defined in Mcloone and Boody. [2] The definition of alcohol unit follows National Health Service (NHS) UK guideline. [3] Inflammatory symptoms were identified as actively having any of these conditions: eczema, rheumatoid arthritis, multiple sclerosis and Crohn's disease. [4] Minor obstetric complications include symphysis pubic dysfunction, chest infection, heartburn, headache, carpal tunnel syndrome, constipation, sciatica, hyperemesis and urinary tract infection.

Table 2

Inter-correlation of mRNA levels of genes regulating fetal glucocorticoid exposure. Correlation analysis was performed from log-transformed QPCR data. In bold, ***p ≤0.001, **p ≤0.01, *p ≤0.05, underlined p ≤0.1. Gc = glucocorticoids.

Table 3

The associations between the levels of placental GC- linked gene and IGF2 family gene expression and maternal mood symptoms are sex specific. Anx= averaged Z score from anxiety symptoms, Dep= averaged Z score, SWLS= Satisfaction with Life Scale. In bold, **p ≤0.01, *p ≤0.05; underline: p ≤ 0.1, ns= not significant. Shadowed cells represent genes which prevent foetal GC exposures. [a]The results in female pool are prior to adjusting for cortisol level at week 36 pregnancy; adjusting for cortisol level did not affect the analyses involving ABCC1 and 11β-HSD1 in female pool.

Demographics			Lean (n = 43)	SO (n = 50)	p
BMI (kg/m^2), mean (SD)			22.83 (1.55)	43.94 (3.71)	***≤0.0001***
Age, mean (SD)			33.96 (4.09)	31.48 (5.41)	***0.016***[a]
Parity, n (%)		0	27 (62.79)	25 (50)	0.295[b]
		≥1	16 (37.21)	25 (50)	
Deprivation category, n (%) [1]		Affluent- intermediate	29 (67.44)	20 (40)	***0.012***[b]
		Deprived- very deprived	14 (32.56)	30 (60)	
Alcohol unit consumed before pregnancy, mean (SD) [2]			7.4 (6.53)	4.09 (4.86)	***0.017***[a]
Smoking during pregnancy, n (%)			2 (4.65)	6 (12)	0.278[b]
Antidepressants, n (%)			2 (4.65)	2 (4)	1[b]
PCOS, n (%)			4 (9.30)	4 (8)	1[b]
Inflammatory symptoms, n (%) [3]			9 (20.93)	14 (28)	0.473[b]
Minor obstetric complications, mean (SD) [4]			2.9 (1.56)	3.39 (1.7)	0.165[a]
Week 17 pregnancy	Anxiety symptoms	HA	5.67 (3.1)	6.1 (3.24)	0.517[a]
		State	30.17 (7.47)	34.84 (9.54)	***0.012***[a]
		Trait	34.14 (9.5)	36.55 (10.23)	0.25[a]
		Averaged z-score	-0.12 (0.87)	0.18 (0.96)	0.128[a]
	Satisfaction with Life Scale (SWLS)		29.95 (6.19)	24.59 (5.27)	0.052[a]
	Depression symptoms	HD	2.29 (2.06)	3.84 (2.76)	***0.004***[a]
		GHQ	1.98 (2.07)	3.35 (2.5)	***0.006***[a]
		Averaged z-score	-0.24 (0.73)	0.23 (0.92)	***0.002***[a]
Week 28 pregnancy	Anxiety symptoms	HA	5.67 (3.06)	6.68 (3.65)	0.157[a]
		State	31.86 (9.2)	35.54 (11.01)	0.089[a]
		Trait	33.9 (8.71)	37.42 (11.91)	0.116[a]
		Averaged z-score	-0.05(0.86)	0.31 (1.12)	0.092[a]
	Satisfaction with Life Scale (SWLS)		27.67 (4.78)	25.04 (5.12)	***0.013***[a]
	Depression symptoms	HD	2.26 (2.05)	4.36 (3.09)	***≤0.0001***[a]
		GHQ	2.29 (2.11)	3.64 (3.20)	***0.021***[a]
		Averaged z-score	-0.32 (0.72)	0.33 (1.13)	***0.002***[a]
Log plasma cortisol		Week 17 pregnancy	3.14 (0.21)	2.97 (0.23)	***0.001***[a]
		Week 28 pregnancy	3.29 (0.31)	3.13 (0.17)	***0.006***[a]
		Week 36 pregnancy	3.30 (0.16)	3.17 (0.20)	***0.002***[a]
Baby gender, n (%)		Male	14 (32.56)	30 (60)	***0.012***[b]
		Female	29 (67.44)	20 (40)	
Mode of delivery, n (%)		Vaginal	27 (62.79)	14 (28)	***0.003***[c]
		Caesarean	16 (37.21)	36 (72)	
Gestational age in days, mean (SD)			281.48 (9.69)	281.12 (9.18)	0.851[a]
Birth weight in grams, mean (SD)		Overall	3446.9 (434.3)	3554.6 (636.6)	0.351[a]
		Male	3449.28 (361.9)	3708.33 (520.5)	0.101[a]
		Female	3445.75 (471.2)	3324 (733.7)	0.482[a]

Table 1

Pearson's Correlation	Gc Retrograde transfer			Gc enzymatic clearance		Gc sensitivity		IGF2, receptor, downstream signal			
	ABCB1	ABCC1	ABCG2	HSD1	HSD2	NR3C1-α	NR3C2	IGF2_1	IGF2_2	IGF2R	mTOR
ABCB1	1										
ABCC1	0.0	1									
ABCG2	0.5***	-0.0	1								
HSD1	0.	0.7***	0.0	1							
HSD2	0.5***	-0.2*	0.4***	-0.1	1						
NR3C1-α	0.1	0.4***	0.2*	0.4***	-0.2*	1					
NR3C2	0.1	-0.0	0.2	0.1	0.1	0.2	1				
IGF2-1	-0.0	0.4***	0.3**	0.4***	-0.1	0.4***	0.4***	1			
IGF2-2	0.1	0.6***	0.1	0.4***	-0.1	0.2*	0.0	0.3***	1		
IGF2R	-0.2*	0.3**	-0.1	0.4***	-0.1	0.3**	0.1	0.3**	0.1	1	
mTOR	0.3*	0.5***	0.3**	0.4***	0.0	0.2*	0.1	0.5***	0.5***	0.1	1

Table 2

Table 3

β (p)	Male (n = 44)						Female (n = 49)					
	week 17			week 28			week 17			week 28		
	Anx	Dep	SWLS	Anx	Dep	SWLS	Anx	Dep	SWLS	Anx	Dep	SWLS
ABCB1	0.21	0.15	-0.24	0.16	0.04	-0.21	-0.20	-0.29	0.48	-0.28	-0.3*	0.55**
ABCC1	-0.29	-0.15	0.30	-0.20	0.03	-0.02	0.17	-0.01	0.32	-0.12	-0.16	0.00
ABCG2	0.11	0.15	-0.31	0.03	0.09	-0.35*	-0.24	-0.37*	0.38	-0.27	-0.29*	0.29*
11β-HSD1	-0.05	0.11	0.15	0.02	0.17	-0.39*	0.16	0.14	0.06	-0.10	-0.16	-0.01
11β-HSD2	0.17	0.21	-0.44*	0.06	-0.16	-0.33	-0.08	-0.11	0.21	-0.20	-0.34*	0.43*
NR3C1-α	-0.01	-0.08	-0.24	-0.14	-0.02	0.07	0.06	0.16	-0.26	0.12	0.29	-0.32*
NR3C2	-0.03	0.02	-0.31	-0.18	-0.03	0.01	0.15	0.23	0.02	0.07	0.01	0.13
IGF2-1[a]	0.07	0.13	-0.05	-0.04	0.10	-0.20	0.14	0.27	-0.24	0.38**	0.4**	-0.38*
IGF2-2	-0.25	-0.15	0.12	-0.24	-0.10	0.02	0.04	-0.10	0.07	-0.11	-0.03	0.03
IGF2R	-0.04	0.00	-0.05	-0.13	-0.10	-0.10	0.19	0.43**	-0.24	0.23	0.27	-0.26
mTOR	-0.24	-0.18	0.16	-0.16	0.01	-0.02	0.13	-0.10	0.24	0.06	0.05	0.11